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Isolation and characterization of a *Chlamydomonas* L-asparaginase

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An L-asparaginase (EC 3.5.1.1) specific for L-asparagine has been purified from a marine *Chlamydomonas* species, the first such enzyme to be purified from a microalga. The purified enzyme (mol.wt. 275000) possessed a K_m for asparagine of 1.34×10^{-4} M and showed limited antitumour activity in an antilymphoma assay *in vivo*. Properties of the enzyme are contrasted with those of asparaginases from prokaryotic and eukaryotic sources.

The enzyme L-asparaginase (E.C. 3.5.1.1) has been intensively investigated over the past two decades owing to its importance as an antineoplastic agent [for reviews, see Wriston & Yellin (1973), Chong & Chang (1977) and Wade (1980)]. Although the enzyme has been found in a variety of bacteria, fungi, yeasts and mammals, few of these purified preparations have possessed antitumour activity (Wriston & Yellin, 1973).

L-Asparaginase has been investigated recently in higher plants on account of the key role L-asparagine plays in the nitrogen nutrition of these organisms (Atkins *et al.*, 1975; Lea *et al.*, 1978; Miflin & Lea, 1977; Sodek *et al.*, 1980).

Although L-asparagine has been known for some time to be an excellent nitrogen source for the growth of eukaryotic microalgae (Ludwig, 1938), the first report of an algal L-asparaginase appeared only fairly recently (Paul & Cooksey, 1979). The organism, a marine *Chlamydomonas* species, could use L-asparagine, but no other amino acid, for autotrophic growth (Paul & Cooksey, 1979). This organism possessed limited capacity to take up L-[^{14}C]asparagine, but deamidated asparagine at the cell surface (Paul & Cooksey, 1979), suggesting an intraperiplasmic location of the L-asparaginase. Nitrogen deprivation stimulated enzyme synthesis (Paul & Cooksey, 1979) and the enzyme was repressed in the presence of combined nitrogen (Paul & Cooksey, 1981*a*). De-repression of synthesis was light-energy dependent and inhibited by cycloheximide and 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (Paul & Cooksey, 1981*a*). Glutamine synthetase showed a similar regulatory pattern in the presence of combined nitrogen, suggesting a common regulatory mechanism for these two enzymes (Paul & Cooksey, 1981*b*).

The present paper reports the first algal L-asparaginase to be purified to near homogeneity and to be tested for antitumour activity in an assay *in vivo*.

Experimental

Materials

Chemicals and substrates were obtained from the following suppliers: L-asparagine, D-asparagine, L-glutamine, L-leucinamide hydrochloride and Tris from Sigma Chemical Co., St. Louis, MO, U.S.A.; L-prolyl-L-leucylglycinamide from Nutritional Biochemicals Corp., Cleveland, OH, U.S.A.; succinamide, butyramide, propionamide, malonamide and carbobenzyloxy-L-asparagine from Aldrich Chemical Co., Milwaukee, WI, U.S.A.; DEAE-Sephacel, Phenyl-Sepharose CL-4B, Sephadex G-200, chromatographically pure ribonuclease A (bovine pancreas), Blue Dextran 2000, ovalbumin and aldolase (rabbit muscle) from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.; chromatographically pure cytochrome *c* (horse heart), chymotrypsinogen A (bovine pancreas), bovine serum albumin, catalase (ox liver) and ferritin from Boehringer-Manheim, Houston, TX, U.S.A. Acrylamide, *NN'*-methylenebisacrylamide, *NNN'N'*-tetramethylethylenediamine, riboflavin, ammonium persulphate and glycine were all electrophoresis grade and obtained from Bio-Rad Laboratories, Rockville Centre, NY, U.S.A. Homogeneous L-asparaginase from *Vibrio succinogenes* [202 units (μmol/min)/mg of protein] was a gift from Dr. John A. Distasio, Department of Microbiology, Uni-

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versity of Miami School of Medicine. Homogeneous *Escherichia coli* L-asparaginase (lot 1028A) was obtained from Mercke, Sharp and Dohme, West Point, PA, U.S.A. L-[U-^{14}C]Asparagine (208 mCi/mmol) and L-[U-^{14}C]glutamine (254 mCi/mmol) were obtained from New England Nuclear Corp., Boston, MA, U.S.A. Hanks buffered salts were obtained from GIBCO, Oakland, CA, U.S.A. All other chemicals were reagent grade and obtained from Fisher scientific Co., Orlando, FL, U.S.A., or J. T. Baker and Co., Phillipsburg, NJ, U.S.A.

Animals and cell lines

Normal C3H mice were obtained from Charles River Breeding Farm, Wilmington, MA, U.S.A., whereas C3H mice bearing the 6C3 HED (Gardner's) murine lymphosarcoma were provided by Dr. Joseph Mayo, National Cancer Institute, Frederick, MD, U.S.A. The *Chlamydomonas* species (Chlorophyta, Volvocales) employed in the present study has been previously described (Paul & Cooksey, 1979).

Production and purification of L-asparaginase

The *Chlamydomonas* species was autotrophically grown in 9.2-litre lots of an artificial-seawater medium as previously described (Paul & Cooksey, 1979). Cultures were harvested by continuous-flow centrifugation (75 ml/min) at 4200*g* at 4°C in a Lourdes Beta-fuge centrifuge. Cell pellets were washed once with 0.15 M-NaCl/10 mM-disodium EDTA and stored at −20°C. The cell pellets of 110.4 litres (49.4 g wet wt. of cells) were resuspended in 0.05 M-$Na_2B_4O_7$ adjusted to pH 8.3 with HCl containing 0.1 mM-disodium EDTA (hereafter termed '0.05 M-sodium borate/0.1 mM-disodium EDTA, pH 8.3') to give a final volume of 132 ml, and passed twice through a French pressure cell as previously described (Paul & Cooksey, 1979). Cell fragments were removed by centrifugation at 4200*g* for 10 min at 4°C, yielding the crude extract. This extract was frozen and thawed at least four times, followed by centrifugation for 10 min at 4200*g* at 4°C (hereafter termed 'freeze-fractionation') to rid the crude extract of photosynthetic membranes and associated proteins.

Finely ground $(NH_4)_2SO_4$ was slowly added with vigorous stirring to the freeze-fractionated supernatant liquid at 0–2°C until a concentration of 1.37 M (37% saturation) was attained. The solution was stirred for an additional 30 min at 0–2°C, followed by centrifugation for 10 min at 4200*g* at 4°C. The supernatant was dialysed against a buffer comprised of 0.05 M-KH_2PO_4/10 mM-NaCl adjusted to pH 7.4 with NaOH at 25°C (hereafter referred to as '0.05 M-KH_2PO_4/NaOH/10 mM-NaCl, pH 7.4') until no NH_4^+ could be detected in the diffusate by Nessler's reagent (Koch & McMeekin, 1924). The enzyme was concentrated by dialysis against poly(ethylene glycol) 20000, followed by dialysis against 0.05 M-KH_2PO_4/NaOH/10 mM-NaCl, pH 7.4, at 4°C overnight. The dialysis residue was added to a 1.5 cm × 50 cm column of DEAE-Sephacel equilibrated with 0.05 M-KH_2PO_4/NaOH/10 mM-NaCl, pH 7.4, and developed with stepwise increments of NaCl (0.01–0.5 M) in the same buffer at a flow rate of 22.9 ml/h under a hydrostatic head of 100 cm. Protein and asparaginase activity was monitored in fractions (1.2 ml each) as described below. The active fractions were pooled, concentrated by dialysis against poly(ethylene glycol) 20000, followed by dialysis against 0.05 M-KH_2PO_4/NaOH/10 mM-NaCl, pH 7.4, at 4°C overnight.

Finely ground NaCl was added to the above dialysis residue to make a final concentration of 4 M. This was added to a 1 cm × 30 cm column of phenyl-Sepharose CL-4B equilibrated with 0.05 M-KH_2PO_4/NaOH/4 M-NaCl, pH 7.4, under a hydrostatic head of 50 cm (21 ml/h flow rate). The column was developed with 0.05 M-KH_2PO_4/NaOH, pH 7.4, containing decreasing concentrations of NaCl (4–1 M) added stepwise, and the active fractions (1.4 ml each) were pooled and dialysed against 0.05 M-KH_2PO_4/NaOH/10 mM-NaCl, pH 7.4.

Protein in the column effluent was monitored at 280 nm with a Model 226 u.v. monitor (Instrument Specialities Co., Lincoln, NE, U.S.A.). Protein in pooled column fractions and other enzyme solutions was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Asparaginase activity was determined as NH_4^+ produced from asparagine by direct nesslerization as previously described (Paul & Cooksey, 1979). One unit of asparaginase is the amount of enzyme that will form 1 μmol of NH_4^+/min at 37°C (Wriston & Yellin, 1973).

Electrophoresis

Disc-gel electrophoresis was performed on 10 cm × 0.4 cm gels as described by Davis (1964). Electrophoresis was performed at 2°C and at 4 mA/gel. For determination of asparaginase activity, gels were measured and sliced into 2 mm slices and each placed in 0.5 ml of 0.05 M-sodium borate buffer (pH 8.3)/0.1 mM-disodium EDTA for at least 24 h to allow activity to diffuse from the gels. Fixed gels were stained with Coomassie Blue (Davis, 1964) or by the silver-staining method of Oakley *et al.* (1980).

Molecular weight

This was estimated on a 1.5 cm × 30 cm column of Sephadex G-200 (48 ml bed vol., 19.2 ml void vol.). A hydrostatic head of 15 cm allowed a flow rate of 8 ml/h. Homogeneous protein standards (see under 'Materials') were dissolved in 0.05 M-KH_2PO_4/NaOH/10 mM-NaCl/0.05% (w/v) sucrose, pH 7.4,

at a final concentration of 10mg/ml, except for ferritin, which was 1mg/ml. A portion (0.5ml) of the partially purified *Chlamydomonas* enzyme (0.93mg of protein, 5units/mg of protein) was added to the column and the elution volume determined as the activity peak.

Stability

The stability of the enzyme in normal human serum (obtained from the National Children's Cardiac Hospital, Miami, FL, U.S.A.) was examined by the method of Broome (1965).

The purified enzyme was assayed in the following buffers, all at 0.05M final concentration at 37°C: acetic acid/sodium acetate (pH4.86, 5.7 and 6.33); K_2HPO_4/KH_2PO_4 (pH6.81, 7.37 and 7.75); $Na_2B_4O_7/HCl$ (pH8.30 and 8.69); and $Na_2B_4O_7/NaOH$ (pH9.52 and 10.01). The purified enzyme was also assayed at various temperatures from 3–55°C in 0.05M-sodium borate (pH8.3)/0.1mM-disodium EDTA.

Effect of cations

To determine the effect of cations on activity, the purified enzyme was dialysed at 4°C exhaustively against 0.02M-Tris adjusted to pH7.6 with HCl at 25°C. The enzyme was assayed in the presence of the cation to be examined (0.05M, as the chloride). Asparaginase activity was determined as described previously (Paul & Cooksey, 1979) except that 25mM-disodium EDTA was added to the 0.1M-trichloroacetic acid 'reaction-stop solution' when assays were performed in the presence of Ca^{2+}, Ba^{2+} and Sr^{2+} to prevent precipitation with Nessler's reagent.

Effect of the reaction end products on activity

The enzyme was assayed as described previously (Paul & Cooksey, 1979) in the presence of 5mM-L-aspartic acid (sodium salt, pH7.0), or in the presence of 1, 10, and 25mM-NH_4Cl, and activity was determined by using the radiochemical assay described by Prusiner & Milner (1970).

Substrate specificity

This was investigated by replacing L-asparagine in asparaginase reaction mixtures with 10mM-D-asparagine, succinamide, butyramide, malonamide, acetamide, propionamide, L-leucinamide, L-prolyl-L-leucylglycinamide, or carbobenzyloxy-L-asparagine, and determining NH_4^+ production with time as previously described (Paul & Cooksey, 1979). Glutaminase activity of the purified L-asparaginase was determined by the radiochemical method of Prusiner & Milner (1970).

Activity as a function of concentration of the substrate L-asparagine and the calculation of K_m was performed as previously described (Paul & Cooksey, 1979), except that the purified enzyme was employed in the place of a cell-free extract.

Determination of Antitumour activity

Male C3H mice (23 in all, 22–26g body wt.) were each subcutaneously implanted with 2×10^6 6C3 HED lymphosarcoma cells in 0.3ml of sterile Hanks buffered salts in the left flank. When tumours reached 11mm in diameter (9days after implantation), six animals received 0.5 units of the *Chlamydomonas* asparaginase (78 units/mg of protein) in 0.2ml of 0.01M-Na_2HPO_4/NaH_2PO_4, pH7.0, twice a day for 4 days (total: 4 units/animal). Control animals received 4 units of the homogeneous *Vibrio succinogenes* L-asparaginase, 4 units of the homogeneous *E. coli* asparaginase or 0.01M-Na_2HPO_4/NaH_2PO_4, pH7.0, on the same regimen. Tumour size was measured in three dimensions daily with calipers, starting 1day before L-asparaginase of buffer treatment.

Results

A typical purification of the *Chlamydomonas* L-asparaginase appears in Table 1. The turbid, dark-green crude extract could be clarified by freezing and thawing followed by low-speed centrifugation to yield the freeze–fractionated supernatant. Preliminary $(NH_4)_2SO_4$ fractionation studies

Table 1. *Summary of the purification of the Chlamydomonas L-asparaginase*

Purification procedures were performed as described in the Experimental section. The starting material was the cell mass (49.4g wet wt.) of 110.4 litres of culture.

Step	Total activity (units)	Specific activity (units/mg of protein)	Purification (fold) Step	Purification (fold) Overall	Yield (%)
Crude fraction	330	0.123	0.0	0.0	100.0
Freeze–fractionation	330	0.491	4.0	4.0	100.0
$(NH_4)_2SO_4$	312	1.41	2.87	11.5	94.5
DEAE-Sephacel	230	9.64	6.84	78.7	69.7
Phenyl-Sepharose	103	78.5	8.15	640.0	31.1

indicated that the enzyme precipitated at 3.12 M (80% saturation) with considerable loss in activity that could not be regained by dialysis (results not shown).

Asparaginase activity was eluted with 0.2 M-NaCl in approximately ten fractions during anion-exchange chromatography on DEAE-Sephacel. The enzyme was eluted during hydrophobic-interaction chromatography on phenyl-Sepharose when the NaCl concentration was reduced to 2 M. After this step the enzyme possessed a specific activity of 78 units/mg of protein and had been purified over 600-fold. Disc-gel electrophoresis indicated the presence of only one band when the gels were stained with Coomassie Blue (Fig. 1*a*), which corresponded to the activity profile (Fig. 1*c*). The more sensitive silver-staining procedure indicated the presence of three faint contaminants as well as the enzyme (Fig. 1*b*).

The molecular weight of the enzyme as determined by gel filtration was 275000, with a 95% confidence interval from 251000 to 295000.

The purified enzyme was stable at room temperature for 24 days in sterile solution. When assayed at various temperatures, the greatest activity occurred at 55°C, the highest temperature examined. An Arrhenius plot of log activity against $1/T$ (in K) resulted in a straight line ($r = 0.99$) down to 10°C (283 K), but activity diminished below this. The energy of activation as estimated from the slope of the line was 42.13 kJ (10.08 kcal)/mol.

Incubation of the enzyme in human serum at

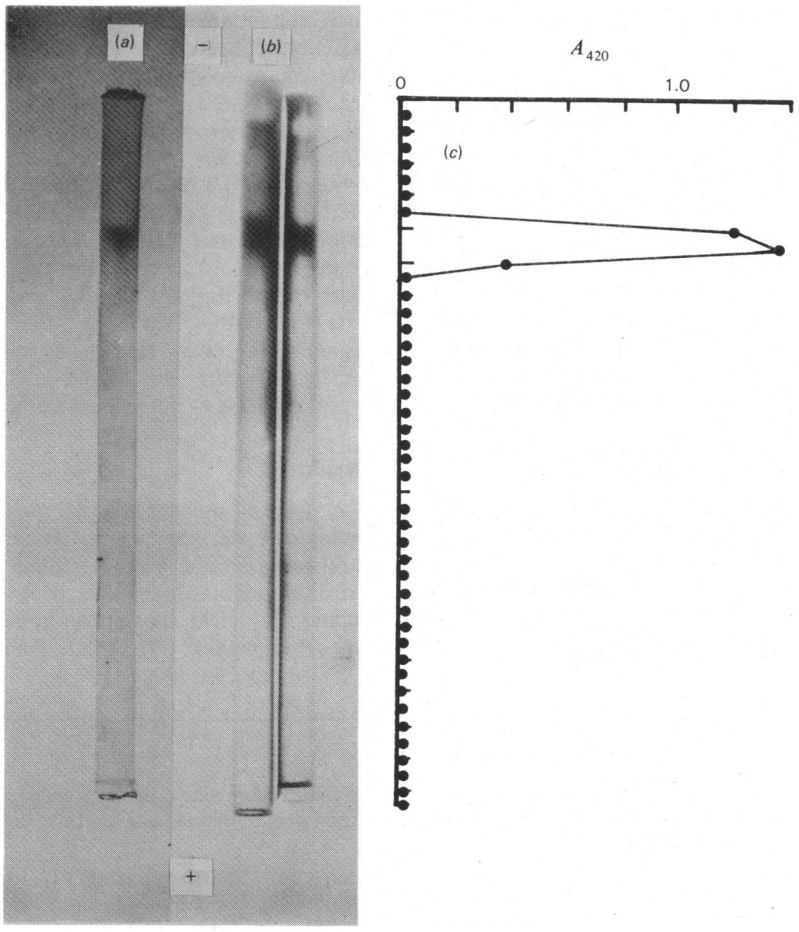

Fig. 1. *Polyacrylamide-gel electrophoresis of the purified Chlamydomonas* L-*asparaginase*
The purified enzyme (78 units/mg of protein) was electrophoresed in 7% (w/v) polyacrylamide as described in the Experimental section. (*a*) Gel stained with Coomassie Blue; (*b*) replicate silver-stained gels by the method of Oakley *et al.* (1980); and (*c*) activity (A_{420} of Nessler-reagent-positive material resulting from incubation with L-asparagine) in gel slices of unstained, unfixed gels.

37°C for 24h resulted in no loss of activity, indicating the absence of any inactivating material in normal human serum.

Unlike the enzyme activity in crude extracts (Paul & Cooksey, 1979) the purified enzyme maintained optimal activity over a wide range of pH (6.8–9.52).

The univalent cations Na^+, K^+ and Li^+ all had a slight stimulatory effect on the L-asparaginase that had been exhaustively dialysed against 0.02M-Tris/HCl, pH 7.6 (131, 122, and 114% of control). Dialysis of the enzyme against EDTA resulted in no loss of activity, suggesting no requirement for bivalent cations. Ba^{2+} and Sr^{2+} had little effect on activity, whereas Ca^{2+} inhibited activity by 25% (Mn^{2+} and Mg^{2+} could not be assayed owing to their interference with direct nesslerization).

Of the asparagine analogues and amides investigated (see the Experimental section), only D-asparagine was hydrolysed (11% of the rate of hydrolysis of the L-isomer).

The products of the asparaginase reaction, aspartate and NH_4^+, did not significantly inhibit the enzyme at concentrations as high as 25mM (NH_4^+) or 5mM (aspartate).

Kinetic analysis of activity against substrate concentration yielded a K_m for asparagine of 1.34×10^{-4}M.

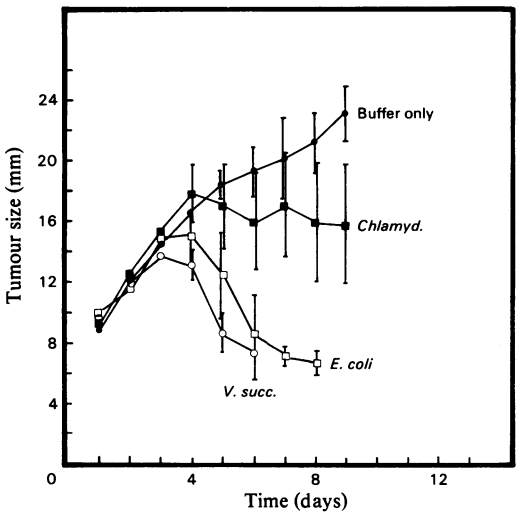

Fig. 2. *Antitumour activity of the Chlamydomonas L-asparaginase*

C3H mice were implanted with the 6C3 HED murine Lymphosarcoma in the left flank. When tumours reached 11mm diameter (day 2 on Figure) animals received 0.5 unit of the *Chlamydomonas* (*Chlamyd.*) (■), *E. coli* (□), or *Vibrio succinogenes* (*V. succ.*) (○) L-asparaginase or 0.01M-phosphate buffer (Buffer only) (●) twice a day for 4 days. Each point represents the mean ± S.D. of tumour measurements on four to six animals.

The results of a typical antitumour assay appear in Fig. 2. In mice treated with the *E. coli* and *Vibrio* asparaginases, potent antitumour agents (Mashburn & Wriston, 1964; Distasio *et al.*, 1977), tumours regressed rapidly to a point where they were no longer palpable (6 and 8 days after treatment with the *Vibrio* and *E. coli* enzymes respectively). Mice treated with an equivalent amount of the *Chlamydomonas* asparaginase showed only slight remission to tumours when compared with controls.

Discussion

An L-asparaginase from a *Chlamydomonas* species has been purified to near homogeneity (78 units/mg of protein) as determined by disc-gel electrophoresis. The present paper reports the first purification and characterization of an L-asparaginase in freshwater or marine microalgae.

The molecular weight of the *Chlamydomonas* L-asparaginase is approximately twice that of prokaryotic asparaginases (130000–150000; Distasio *et al.*, 1976; Kitto *et al.*, 1979; Wade *et al.*, 1968; Resnick & Magasanick, 1976). The molecular weights of higher-plant asparaginases purified from lupin (*Lupinus polyphyllus*) seeds and pea (*Pisum sativum*) seeds were found to be 72000 (Lea *et al.*, 1978) and 68000 (Sodek *et al.*, 1980) respectively, or approximately one-fourth that of the *Chlamydomonas* enzyme.

In common with the *E. coli* L-asparaginase isoenzyme EC 2 (Wriston & Yellin, 1973), the *Chlamydomonas* enzyme shows some degree of thermal stability, and possesses optimum activity over a wide range of pH values. As found with other asparaginases (Wriston & Yellin, 1973), there is no bivalent-cation requirement and no inhibition by the reaction end products, aspartate and NH_4^+. These are characteristics common to extracellular enzymes or enzymes found in the periplasmic space, where conditions of ionic strength, pH and substrate concentration are poorly controlled (Wade *et al.*, 1971). Previous studies suggest that the *Chlamydomonas* asparaginase is associated with the cell surface (Paul & Cooksey, 1979), as are many other microbial asparaginases (Wade, 1980). The fact that the enzyme maintained solubility at very high ionic strength [80% satd. $(NH_4)_2SO_4$] and its ability to tightly bind to phenyl-Sepharose suggest a somewhat hydrophobic character, which may enable it to interact with the cell membrane. The enzyme has been shown not to be produced extracellularly (Paul & Cooksey, 1979).

Unlike the *E. coli* enzyme, both the *Chlamydomonas* and an L-asparaginase isolated from *Saccharomyces cerevisiae* (Pauling & Jones, 1980) were repressed by combined nitrogen; de-repression

requires an energy source and is inhibited by cycloheximide (Paul & Cooksey, 1981*a*).

The *Chlamydomonas* asparaginase showed only slight stimulation of activity in the presence of univalent cations. Sodek *et al.* (1980) described an L-asparaginase from pea seeds with an absolute requirement for K^+. Assay mixtures containing only Tris possessed 5.6% of the activity of those containing K^+, whereas assay mixtures with Na^+ or Rb^+ only possessed 36–38% of the activity. The stimulation of activity of the *Chlamydomonas* asparaginase by univalent cations was slight in comparison with that observed for pea seeds, and does not possess the same specificity for K^+.

Prokaryotic L-asparaginases can generally hydrolyse glutamine and other asparagine analogues (Wriston & Yellin, 1973). Preparations of asparaginase from *E. coli*, *Erwinia carotovora* and *Acinetobacter* possessed between 5 and 10% glutaminase activity (Wriston & Yellin, 1973; Wade *et al.*, 1971; Joner *et al.*, 1973), whereas glutaminase activities of several *Pseudomonas* enzymes were 145–200% of the asparaginase activity. Only one prokaryotic asparaginase has been found to possess negligible glutaminase activity (Distasio *et al.*, 1976). Glutaminase activity in asparaginase preparations has been implicated as a cause for immunosuppression and other undesirable side effects of asparaginase therapy (Durden & Distasio, 1981).

Unlike the prokaryotic enzymes, asparaginases found in higher plants generally do not hydrolyse glutamine, as was found with the *Chlamydomonas* enzyme. Since the major assimilatory pathway of inorganic nitrogen is through glutamine via glutamine synthetase (Miflin & Lea, 1977), the presence of glutaminase would result in a futile cycle in which NH_3 would be liberated and assimilated at the expense of ATP (Lea *et al.*, 1978).

The *Chlamydomonas* L-asparaginase possessed little ability to inhibit the growth of the Gardner's lymphosarcoma in C3H mice. Important requirements for antitumour activity include good activity at physiological pH and temperature, no inhibition of the enzyme by reaction end products, no cofactor requirement, slow rate of clearance from the serum and relatively low K_m (Holcenberg & Roberts, 1977; Uren & Handschumacher, 1975). Although the *Chlamydomonas* asparaginase has satisfied many of these requirements, its K_m is higher than asparaginases employed in chemotherapy (Wriston & Yellin, 1973). Broome (1968) pointed out that serum asparagine levels must be reduced below 10^{-5} M to limit protein synthesis *in vivo* and thereby inhibit tumour growth. Kinetic studies with this enzyme indicate that at 10 μM-L-asparagine the velocity is only 10% of the V_{max}. Thus the relatively high K_m may have limited the antitumour activity of this enzyme, although other factors such as serum survival were not investigated.

The physiological role of this enzyme in the metabolism of the *Chlamydomonas* species remains unclear. Although an acetamidase has been recently described in *Chlamydomonas reinhardi* that can hydrolyse several simple amides, this enzyme possesses no activity toward L-asparagine (Gresshoff, 1981). It is unlikely that an enzyme extremely specific for L-asparagine with a K_m of 10^{-4} M as found in this organism could deamidate appreciable levels of asparagine in the marine environment, where concentrations of most amino acids are generally less than 1 μM (North, 1975).

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